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(54) PROCESS FOR THE ENZYMATIC PREPARATION OF BASIC FIBROBLAST GROWTH FACTOR

VERFAHREN ZUR ENZYMATISCHEN HERSTELLUNG VON BASISCHEN FIBROBLASTEN-WACHSTUMSFAKTORS

PROCEDE DE PREPARATION ENZYMATIQUE DU FACTEUR DE CROISSANCE FIBROBLASTIQUE DE BASE

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Exhibit 1

Description

The present invention relates to the enzymatic preparation of basic fibroblast growth factor (bFGF).

bFGF was originally isolated from brain and pituitary as a polypeptide of 146 amino acids (Esch et al, PNAS USA 82, 6507-6511, 1985). The gene for bovine bFGF has been cloned (Abraham et al, Science, 233, 545-548, 1986). The nucleotide sequence predicted a 155 amino acid bFGF translation product. Further work has shown that a 154 amino acid bFGF can be extracted together with a 146 amino acid bFGF from normal pituitary tissue upon addition of enzyme inhibitors (Ueno et al, Biochem. Biophys. Res. Comm. 138, 580-588, 1986) and that acid proteases in brain and hepatoma cells cleave bFGF (Klagsbrun et al, PNAS USA 84, 1839-1843, 1987).

Protein engineering techniques have allowed the availability of recombinant growth factors for therapeutic use. However, once they have been expressed these growth factors could be processed into a mixture of different forms. FGFs are no exception in this respect (Barr et al, J. Biol. Chem. 263, 31, 16471-16478, 1988).

We have now devised a process for the preparation of a bFGF which is truncated at its N-terminus. This process can be applied to obtain the 146-amino acid form of bFGF from longer forms and to produce a single form of bFGF from a mixture of bFGFs. The resulting form is pure and is not contaminated by other forms of bFGF.

Accordingly, the present invention provides a process for the preparation of a bFGF, which process comprises:

(i) forming an adduct between heparin or heparan sulphate and a bFGF which has the 9-10 Leu-Pro bond;
(ii) treating the adduct with pepsin A or cathepsin D, thereby cleaving the said bond; and
(iii) releasing from the adduct the bFGF thus obtained.

A mixture of two or more bFGFs which each have the 9-10 Leu-Pro bond, which have the same amino acid sequence from position 11 to their C-termini and which have different N-terminal amino acid sequences may be employed in step (i). Their amino acid sequences may differ only by having different N-termini. Typically, the only difference between the sequences of the bFGFs is that the number of N-terminal amino acid residues is different. One bFGF may have one or more additional N-terminal amino acid residues than another bFGF. Alternatively, the N-terminal amino acid sequences before position 9 of each bFGF may comprise different amino acid residues.

It is therefore possible to apply the present process to any mixture of bFGFs where the amino acid sequence of each bFGF in the mixture starts with a N-terminal amino acid residue numbered lower than 9 and the bFGFs have different N-termini. The mixture employed in step (i) may therefore be composed of bFGFs having the general formula:

$$NH_2\text{-}(AA)_x\text{-Leu-Pro-Y-COOH}$$

wherein AA is any amino acid residue, x is zero or an integer and Y denotes the amino acid sequence of (11-155) bFGF. Full length bFGF has 155 amino acid residues and can be designated 155-bFGF or (1-155)bFGF. The amino acid sequence of human (1-155)bFGF is shown in SEQ ID NO: 1. The invention can therefore be applied to one of, or a mixture of two or more of, (1-155)bFGF to (8-155)bFGF in which case x in the formula above is an integer of from 8 to 1 respectively and $(AA)_x$ denotes sequence shown one of SEQ ID NOS: 2 to 6, IleThrThr, ThrThr or Thr. In particular, the invention can be applied to a mixture of 154 amino acid residue bFGF[(2-155)bFGF] and 153 amino acid residues bFGF[(3-155)bFGF].

The bFGF or the mixture of bFGFs is generally obtained by recombinant DNA techniques. Different forms of bFGF are obtained in such mixtures due to processing of the translation product at its N-terminus. The or each bFGF may be a human, murine or rodent bFGF.

An adduct may be formed between a protecting agent for bFGF selected from heparin and heparan sulphate, and the mixture of bFGFs in any convenient fashion. The protecting agent and bFGFs are typically provided in an aqueous solution. This solution may be buffered. An antioxidant such as dithiothreitol may be present to prevent protein oxidation. The ratio of bFGF:protecting agent may be from 0.5:1 to 10:1 (w/w), for example from 1:1 to 5:1 (w/w). The protection of the bFGFs as adducts prevents further unwanted hydrolysis when pepsin A is added.

Pepsin A (EC 3.4.23.1) or cathepsin D (EC 3.4.23.5) is next contacted with the adduct. This results in specific cleavage of the 9-10 Leu-Pro bond in the bFGFs held in the adducts. The enzyme may be provided in a solution of the adduct. The enzyme may therefore be added to a solution of the bFGFs and the protecting agent. The enzyme is generally provided in a solution adjusted to pH 4 to 6.

The solution is incubated for, for example in the case of pepsin A, from 30 minutes to 10 hours, for example from 1 to 8 hours. Incubation is typically longer for cathepsin D, for example from 90 to 130 hours, suitably about 110 hours. The incubation temperature may be from 5°C to room temperature, for example about 10°C. Incubation is carried out until reaction is complete.

Alternatively, the protecting agent may be immobilised on a support to form an affinity column. Any appropriate

support may be used, such as an agarose gel or cross-linked dextran gel beads (for example Sepharose). A solution of the bFGFs is loaded onto the column. The bFGFs bind to the protecting agent and are held on the column. A solution of enzyme may then be passed through the column. Finally, the truncated single form of bFGF may be eluted from the column. This may be achieved with a linear gradient of aqueous sodium chloride solution.

A pure form of bFGF can be obtained by way of the present invention. In particular, the 146 amino acid form of bFGF designated (10-155) bFGF can be obtained. The bFGF obtained can be used to treat wounds and burns, for example. The bFGF may be applied to a wound or burn in any suitable formulation. Such formulations therefore typically further comprise a physiologically acceptable carrier or diluent.

The following Examples illustrate the invention. A Preparation Example and Comparative Examples are provided.

Preparation Example: Preparation of 154/153 form of bFGF

The construction of the synthetic DNA sequence for b-FGF and of the expression plasmid carrying such sequence was performed according to the procedure described in EP-A-363675. The fermentation and purification process was carried out as follows:

(a) Fermentation process

A bacterial strain, E. coli type B, from the Institute Pasteur collection, was transformed with a plasmid carrying both the human gene coding for bFGF and the gene for tetracycline resistance. This transformed strain was used for the production of recombinant non-glycosylated h-bFGF (human bFGF). A Master Cell Bank (15 freeze-dried vials) and a Working Cell Bank (W.C.B.) (70 vials stored in liquid nitrogen at -190°C) of this strain were prepared. The content of one vial of W.C.B. was used as the inoculum for the fermentation phase.

The fermentation process was carried out in 10 1 fermentors filled with 4 1 of culture medium. Tetracycline hydrochloride was added to the medium in order to maintain the conditions of strain selection. After 20 hours of growth at 37°C the final biomass was 42±2 g/l dry weight, and the production of bFGF was 2500 ± 500 mg/l as measured by comparative gel electrophoresis.

Enrichment in pure oxygen was required during the fermentation phase in order to allow a large bacterial growth.

(b) Initial purification

The cells (microorganisms) were separated from the total fermentation broth by centrifugation. The resulting pellet was resuspended in a sodium phosphate buffer containing sodium chloride. A minimum of 3 passages through a high pressure homogenizer were necessary for efficient cell breakage. The resulting cell lysate was clarified by centrifugation and the supernatant was collected for further processing.

(c) Purification

The clarified supernatant was loaded on a column of Sepharose (Trade Mark) S Fast Flow (cation exchanger) and the product was eluted from this column using a gradient of increasing sodium chloride concentrations in a phosphate buffer. The product was further purified on a column of Heparin Sepharose 6 B by eluting with a gradient of increasing sodium chloride concentration in a phosphate buffer. Finally a buffer exchange was made on a Sephadex (Trade Mark) G25 resin to obtain the product in the bulk product buffer (Sodium phosphate -EDTA).

(d) Column sanitization

Sepharose S Fast Flow and Sephadex G25 columns were sanitized by washing with sodium hydroxide solutions. Heparin Sepharose was washed alternatively with solutions at pH = 8.5 and pH = 5.5 containing 3M sodium chloride.

In this way, a 154/153 form of bFGF was obtained. This is an approximately 50:50 mixture of:

- a 154 amino acid human bFGF [(2-155)bFGF] having the amino acid sequence of the 155 amino acid form which is reported by Abraham et al and shown in SEQ ID NO: 1 but without the N-terminal Met residue; and
- a 153 amino acid human bFGF [(3-155)bFGF] consisting of the 155 amino acid form of bFGF shown in SEQ ID NO: 1 but without the N-terminal Met and Ala residues.

EXAMPLE 1: Heparan sulphate as protecting agent; pepsin A

1. Preparation of the protein sample:

The 154/153 form of bFGF of the Preparation Example was provided at a concentration of 1.8 mg/ml in a buffered solution:

- 10 mM monosodium phosphate,
- 0.1 mM EDTA disodium salt,
- pH 6.0.

To avoid any protein oxidation during the controlled hydrolysis, 20 mg of dithiothreitol were added to 1.8 mg of bFGF.

2. bFGF enzymatic hydrolysis procedure:

a) Standard solution of protecting agent

Solution 1 : 20 mg of protecting agent in 1 ml of H_2O

b) Preparation of solution of bFGF and protecting agent

- 100 µl of the protein sample,
- 9 µl of solution 1,
- 3 µl 1 M sodium citrate, pH 3.0,
- 70 µl H_2O,
- 18 µg (18 µl) of pepsin A from porcine intestinal mucosa
- Final volume: 200 µl

The protecting agent was heparan sulphate and the bFGF/protecting agent ratio was 1:1 (w/w). Pepsin A (EC: 3.4.23.1) was added to the solution of bFGF and protecting agent at pH 4.0 and incubated at 10°C for 1 hour.

3. Kinetics of the enzymatic hydrolysis:

The hydrolysis procedure above was followed at different times by SDS-PAGE analysis. Samples of the digestion mixture were analysed after 1, 20, 40 and 60 minutes by SDS-PAGE. Separation of the 154/153 bFGF form from the 146-bFGF form was achieved in the SDS-PAGE analysis using the Phast System (Trade Mark; Pharmacia). With regard to the sample buffer, sample preparation conditions were defined as follows in order to avoid any protein precipitation of the sample in the stacking zone of the Phast Gel High Density:

- 15 µl of the protein solution,
- 10 µl of 40 mM Tris/HCl, 4 mM EDTA, 10 % SDS, 20 % mercaptoethanol, pH 8.0,
- 3 µl of DMSO (dimethyl sulphoxide), and
- 1 µl of Bromophenol blue (0.3 %).

Sample denaturation was achieved by heating the sample at 100°C for 5 minutes. The analysis of the samples showed that, in the sample taken from the digestion mixture after one hour, the 154/153-bFGF fragment had completely disappeared. One major fragment was obtained, which corresponded exactly to the 146-bFGF recombinant fragment used as reference.

4. Purification of bFGF

The reaction mixture was directly loaded onto a Heparin-agarose column preequilibrated with Tris buffer 10mM, pH 8.0, 0.5M NaCl. bFGF was eluted at 1.5M NaCl using a gradient of 0.5M to 3M NaCl. Protein concentration was determined by the Bradford method using 154/153 bFGF as reference.

5. Electroblotting of the 146-form of bFGF

The procedure used in this electroblotting analysis was similar to that described by Ploug et al (Anal. Biochem.

181, 33-39, 1989). The membrane used was Immobilon PVDF (Millipore).

After electrophoresis, the gel was equilibrated in a transfer buffer (10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), pH 11.0, 20 % Methanol) for 10 min. The Immobilon PVDF was initially wetted with pure methanol and then equilibrated in the transfer buffer before use. A semi-dry blotting assembly was used (Kyhse-Andersen, J. Biochem. Biophys. Methods 10, 203-209, 1984). The electrotransfer was performed for 2 h at 0.2 mA/cm^2.

The PVDF membrane was rinsed in water and then stained in 0.1 % (w/v) Coomassie brilliant blue R-250 in 50 % (v/v) methanol for 1 min. Excess dye was removed by a brief wash with water followed by destaining in 40 % (v/v) methanol including 10 % (v/v) acetic acid for 5-10 min. The PVDF membrane was then carefully rinsed in water before sequencing.

6. Amino-terminal sequence analysis

Cut areas of the PVDF membrane containing the stained protein were placed as a single layer on top of a Polybrene-conditioned filter. Sequencing was performed on an Applied Biosystems sequencer Model 470 A equipped with an on-line PTH-amino acid analyzer Model 120 A.

The first three amino acids of the NH_2-terminal part of the protein determined under these conditions were:

Pro - Ala - Leu

These amino acids correspond to the three amino acids of the amino-terminal 146-bFGF form.

7. Laser scan densitometry analysis

This analysis was carried out in order to determine the percentage of 146-bFGF produced after one hour of hydrolysis of 154/153-bFGF by pepsin A. After 1 h, 50 µl of the reaction mixture were mixed to 50 µl of SDS-denaturating buffer. In the same conditions, bFGF samples of different protein concentrations were SDS-denaturated. These samples were separated by electrophoresis on a gel PAA 4/30 (Pharmacia). The densitometry of each peak was analyzed using LKB BROMA 2220 recording integrator. Using the standard curve obtained in these conditions, it was found that the concentration of 146-bFGF form corresponded to 91% of the starting 154/153-bFGF concentration.

8. 146-bFGF yield after affinity purification

The yield of the enzymatic hydrolysis reaction was also determined after purification of the 146-bFGF form onto a heparin-agarose column. 1.64 mg of the reaction mixture from 2. above were loaded onto a heparin-agarose column preequilibrated with Tris 10 mM, pH 8, 0.5 M NaCl. At pH 8, pepsin A is not at all active and the complex between heparan sulfate and bFGF is destabilized, allowing the absorption of bFGF onto heparin-agarose present in excess in the medium. bFGF was eluted at 1.5 M NaCl. 1.14 mg of the 146-bFGF form was recovered.

The overall yield of the obtention of the 146-bFGF amino acid form after controlled hydrolysis using heparan sulfate as protecting agent and affinity column purification was 73%.

EXAMPLE 2: Heparin as protecting agent; pepsin A

The procedure of Example 1 was repeated except that the protecting agent was heparin, the bFGF-heparin ratio was 5:1 (w/w), aliquots of the digestion mixture were analysed after 2 minutes and 4, 6 and 8 hours and the total incubation ime was 8 h. The solution of bFGF and heparin consisted of:

- 100 µl of the protein sample,
- 18 µl of solution 2,
- 3 µl of 1 M sodium citrate, pH 3.0,
- 70 µl of H_2O,
- 18 µg of pepsin A,
- final volume: 200 µl

Solution 2 consisted of 100 µl of solution 1 and 900 µl of H_2O. Under these conditions, only one fragment was also obtained after 8 hours incubation. This fragment had an apparent molecular weight of 16,200, as determined by SDS-PAGE analysis.

By amino-terminal sequence analysis, the same three N-terminal amino acids as in Example 1 were obtained:

Pro - Ala - Leu

EXAMPLE 3: Heparin as protecting agent; pepsin A

The yield of the controlled hydrolysis of bFGF protected by heparin was also determined after purification of the 146-bFGF fragment onto a heparin-agarose column. After 7 h of hydrolysis in the same conditions as in Example 2, the reaction medium containing 1.54 mg of bFGF was loaded onto a heparin-agarose column preequilibrated with 10 mM sodium phosphate buffer, pH 8.0 and 0.5 M NaCl. 1.02 mg of 146-bFGF was eluted at 1.5 M NaCl.

The overall yield of the obtention of the 146-bFGF amino acid form after affinity column purification using heparin as protecting agent was 69%.

EXAMPLE 4: Heparin immobilised on Agarose as protecting agent: pepsin A

1. bFGF enzymatic hydrolysis procedure

A column of heparin-agarose (7 ml gel containing 5.6 mg of heparin) was equilibrated with sodium monophosphate 10 mM, 0.1 mM EDTA, NaCl 0.5 M, pH 4.0 at 10°C. The 154/153-bFGF was loaded on the affinity column. Then 0.18 mg of pepsin A diluted in the same phosphate solution was loaded on the top of the column. The enzyme is recirculated throughout the column at a 0.5 ml/min flow rate for 6 h.

At t=6 h, the column was washed with 10 mM sodium phosphate buffer pH 7.0, 0.1 mM EDTA, 0.5 M NaCl, (3 volumes), then with a 0.5 M to 3 M NaCl gradient. bFGF was eluted at 1.5 M NaCl.

2. Amino-terminal sequence analysis

After electrophoresis, the gel was electroblotted on Immobilon PVDF using a semi-dry blotting assembly as described in Example 1. Cut areas of the PVDF membrane containing the stained protein were placed as a single layer on top of a Polybrene-conditioned filter. The first three amino acid of the NH2-terminal part of the protein determined under these conditions were:

Pro - Ala - Leu

These amino acid correspond to the three amino acids of the amino-terminal part 146-bFGF form.

3. 146-bFGF recovery yield

The yield of the hydrolysis reaction was determined after the quantitative determination of the 146-bFGF fragment rinsed out of the heparin-agarose column. 1.28 mg of 146-bFGF was recovered. The overall yield of the obtention of the 146-bFGF amino acid form (Mw=16,200) after controlled hydrolysis onto a heparin-agarose column was 81%.

EXAMPLE 5: Heparin as protecting agent; cathepsin D

1. bFGF enzymatic hydrolysis procedure

Example 2 was repeated except cathepsin D from bovine spleen was used instead of pepsin A and the incubation time was 110 h.

2. Kinetics of the reaction

The appearance of the 146-bFGF fragment obtained during the enzymatic hydrolysis is very low but was detected by SDS-PAGE analysis after 1 h. After 72 h, the 154/153-bFGF hydrolysis was not complete and 36 µg of cathepsin D was added in the reaction medium. 40 h later, the reaction was complete. This is due to low activity of cathepsin D (10 units/mg).

3. **Amino-terminal sequence analysis**

After electrophoresis, the gel was electroblotted on Immobilon PVDF using a semi-dry blotting assembly as described in Example 1. Excised areas of the PVDF membrane containing the stained protein were placed as a single layer on top of a Polybrene-conditioned filter. Sequencing was performed on an Applied Biosystems sequencer analysis Model 470A equipped with an on-line PTH-amino acid analyzer Model 120A. The first three amino acid of the NH2-terminal part of the protein determined under these conditions were:

Pro - Ala - Leu

Comparative Example 1: Pepsin A without protecting agent

Example 1 was repeated with the exception that no protecting agent was present. The 154/153 form of bFGF was completely digested by the pepsin A within a few minutes.

Comparative Example 2: Cathepsin D without protecting agent

Example 5 was repeated with the exception that no protecting agent was present. The 154/153 form of bFGF was completely digested by the cathepsin D within a few minutes.

Comparative Example 3: α-Chymotrypsin

Example 1 was repeated except that α-chymotrypsin was employed in place of pepsin A and no protecting agent was present. The 154/153 form of bFGF was completely digested by the α-chymotrypsin within a few minutes. Different anionic compounds were therefore tested for their ability to protect the bFGF from α-chymotrypsin:

1. **Heparin 3,000 (Sigma, H 7516)**

- bFGF/protect;ng agent ratio = 1
- temperature = 10°C
- pH = 7.5
- incubation time = 4 h

Using heparin 3,000 as protecting agent, one fragment of apparent molecular weight of 14,000 was obtained mainly and some other fragments of lower molecular weight, as determined by SDS-PAGE analysis.

2. **Heparin (Sigma, H 3125)**

- bFGF/protecting agent ratio (w/w) = 5
- temperature = 10°C
- pH = 7.5
- incubation time = 4 h

Using this heparin grade as protecting agent, the protection was not suitable and many fragments were obtained.

3 . **Chondroitin sulfate**

- bFGF/protecting agent ratio (w/w) = 1
- temperature = 10°C
- pH = 7.5
- incubation time = 2 h

The protection was not suitable and many fragments were obtained.

4. **Dermatan sulfate**

- bFGF/protecting agent ratio (w/w) = 1

- temperature = 10°C
- pH = 7.5
- incubation time = 2 h

The protection was not suitable and many fragments were obtained.

5. Polyaspartic acid

- bFGF/protecting agent ratio (w/w) = 1
- temperature = 10°C
- pH = 7.5
- incubation time = 2 h

Using polyaspartic acid as protecting agent, the protection was not suitable and many fragments were obtained.

EXAMPLE 6: Heparin or heparan sulphate as protecting agent; pepsin A or cathepsin D.

1. Experimental Protocol

Proteolytic treatment of soluble complexes bFGF-heparin and bFGF-heparan sulfate in solution

1.6 mg of the 154/153-amino acid form of bFGF were complexed to 1.6 mg of heparin or heparan sulfate in 10 mM citrate-phosphate buffer pH 4.0, 10°C. After 1 hour incubation time 160 µg of pepsin A (Sigma P-6887, 3200-4500 units/mg protein) were added to the solution. Samples of the reaction medium were taken at different times and directly loaded onto a heparin-sepharose column preequilibrated with 10 mM phosphate buffer pH 8.0/0.5 M NaCl at 4°C. The column was then washed extensively with the same buffer and the bFGF was eluted with 3 M NaCl in lo mM phosphate buffer pH 8.0. The pooled fractions were desalted on a Sephadex G25 column previously equilibrated in 10 mM phosphate buffer pH 6.0 and subjected to the SDS-PAGE analysis. Using heparan sulfate as protective agent, 146-bFGF was obtained in quantitative yield after 1 hour. Pepsin treatment of bFGF-heparin complex led to the same quantitative yield just in 6.5 hours incubation time.

Pepsin A treatment of bFGF bound to a heparin-Sepharose column

50 mg of bFGF (154/153) were loaded at a flow rate of 2.5 ml/min on a heparin-Sepharose column (Pharmacia) (2.6 x 22.5 cm) previously equilibrated in 25 mM citrate buffer pH 4.0/0.5 M NaCl at 4°C. A solution of 680 units/ml of porcine pepsin A (Sigma) in citrate-phosphate buffer was continuously recycled through the column at 2.5 ml/min for 3 hours at 4°C. The column was then washed extensively with a 25 mM phosphate buffer pH 8.0/0.5 M NaCl to inactivate and to eliminate the enzyme. The bFGF was step-eluted with 3 M NaCl in 25 mM phosphate buffer pH 8.0. The bFGF-containing fractions were pooled, concentrated by ultrafiltration on Amicon PM 10 membrane and desalted on a Sephadex G25 column (Pharmacia) previously equilibrated in 10 mM phosphate buffer pH 6.0.

N-terminal sequence analysis

Automated N-terminal sequence analysis was performed on a Model 477A Pulsed Liquid Phase Sequencer (Applied Biosystems, CA, USA) with on-line Model 120A PTH-Anlayzer. Normal-1 program with little modifications was used. All sequencing materials and reagents were purchased from Applied Biosystems.

C-terminal sequence analysis

Time-course study of carboxypeptidase P digestion of bFGF was performed at room temperature in 10 mM sodium acetate buffer pH 3.8/0.05% Brij-35, using an enzyme to substrate ratio of approximately 1:100 (w/w) (Lu et al, J. Chromatogr. 447, 351-364, 1988).

Digestions of 0.5-1 nmol of protein were carried out for two hours with 0.1-0.2 µg of CpP (Boehringer); N-Leucine was added as an internal standard. At different times 10/100 µl aliquots were withdrawn and subjected to amino acid analysis after automated derivatization with PITC on a Model 420A Derivatizer (Applied Biosystems) and subsequently injected into a RP-HPLC with on-line Model 130A analyzer. Separation of the derivatized PTC-ami:.o acids was achieved on a PTC-C8 column (P/N 0711-0204, 220 x 2.1 mm, 5 µ, Applied Biosystems).

Bioassays

An endothelial cell-strain derived from bovine aorta (BAEC) was used to study the proliferative response induced by bFGF. Cells were plated at 2500 cells/well in 96 well microtitre plates in complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 13% fetal bovine serum (FBS) (Gibco UK). After attachment, complete medium was replaced with experimental media consisting of DMEM supplemented with 0.5% fetal calf serum (FBS), 0.1% bovine serum albumin (BSA) (Sigma USA) and the desired concentrations of bFGF (Erbamont). The cultures were incubated for 3 days at which time they were fixed with formalin and stained with 0.5% crystal violet. After staining wells were thoroughly washed to remove unincorporated dye. Methanol (95%; 0.1 ml/well) was added to each well to extract the dye to an extent proportional to the amount of cells grown per well. Plates were transferred for automatic reading to a spectrophotometric microplate reader equipped with a 540 nm filter.

For the synthesis of plasminogen activator, BAEC (3 x 10^4 cells in 0.2 ml/well) were seeded in 96 well microtitre plates in complete growth medium that was replaced after attachment with DMEM supplemented with 0.5% FBS, 0.1% BSA and the test concentrations of bFGF. After incubating for 28 h, cultures were washed and cells were lysed with a solution containing 0.5% Triton X-100. Aliquots of the cell-lysates were assayed for plasminogen activator activity using a chromogenic substrate (Spectrozyme PL) and plasminogen (both reagents from American Diagnostica Inc.) for the amidolytic assay.

2. Results

Controlled enzymatic processing of bFGF

The purified recombinant 154/153 mixture was incubated with two different aspartic proteases: pepsin A and cathepsin D. Aliquots of the reaction mixture were taken at various time intervals and submitted to SDS-PAGE analysis showing that, in the absence of any protecting agent, bFGF was quickly digested into small peptides. On the contrary, the treatment of bFGF (154/153) with pepsin A (10:1 w/w; pH 4.0; 10°C) in the presence of heparin or heparan sulfate (1:1 w/W), resulted in the progressive and complete conversion of the 154/153 amino acid forms to a lower molecular weight form which comigrated with our 146/145 amino acid form standard.

After electroblotting on a PDVF membrane the low molecular weight band that resulted from the enzymatic digestion was submitted to automated N-terminal sequence analysis on a pulsed liquid phase sequence . The first three cycles resulted in a single homogeneous sequence; Pro-Ala-Leu that corresponds to the intact N-terminal end of the known 146-amino acid form. No other sequence was detected showing that, despite of the presence of three Leu-Pro sites on the bFGF molecule, when the elongated forms of bFGF were complexed to heparin or to heparan sulfate, the digestion with pepsin A cleaved specifically and only the Leu_9-Pro_{10} peptide bond.

A controlled proteolytic cleavage of the "heparin-protected" NH_2-extended bFGF molecule as obtained with pepsin A, was achieved after digestion with cathepsin D, although a longer incubation time was required for this proteolytic reaction, possibly due to the lower specific activity of the enzyme preparation used. When chymotrypsin was added under similar conditions but at pH 7.5, a single polypeptide of about 14000 dalton was detected after gel electrophoresis of the incubation mixture with no evidence of the presence of a 146 amino acid form.

Large scale enzymatic processing of bFGF on heparin-Sepharose column

The results obtained in solution and in small batch reactions were verified on a larger scale process with the 154/153 mixture of elongated bFGF bound to a herparin-Sepharose column. Accordingly, 50 mg of bFGF (154/153) were loaded on a heparin-Sepharose column previously equilibrated in citrate-phosphate buffer pH 4.0. A solution of pepsin A was recycled continuously through the column for 3 hours at 4°C. Thereafter the column was washed with a phosphate buffer at alkaline pH both to inactivate and to eliminate the enzyme. The resulting polypeptide was eluted from the column with 3 M NaCl in phosphate buffer at pH 8.0. The bFGF-containing fractions were collected and desalted on a Sephadex G25 column.

The pooled fractions analyzed on SDS-PAGE showed a single band with a molecular weight corresponding t- that of the standard 146/145 bFGF form. Reverse-phase HPLC analysis resulted also in a single peak. N-terminal sequence analysis yielded a single sequence corresponding to the correct, intact N-terminal end of the 146-amino acid form, i. e. Pro-Ala-Leu-. C-terminal sequence analysis showed the expected carboxy-terminal end of the bFGF molecule, i.e. -Ala-Lys-Ser. Thus, also when bFGF was bound to the heparin-Sepharose resin, pepsin A was able to cleave specifically the molecule at the Leu_9-Pro_{10} bond generating a homogeneous 146-amino acid form.

In vitro bioassays of the bFGF forms

The biological activity of the mixture of the 154/153-amino acid form was compared to that of the homogeneous 146-amino acid form obtained by the proteolytic process described. Two activities, the induction of a proliferative response and the synthesis of plasminogen activator, were studied in bovine aortic endothelial cells (BAEC). Both assays confirmed the in vitro biological equivalence of the 154/153- as compared to the 146-amino acid form obtained by the enzymatic process.

SEQUENCE LISTING

(1) INFORMATION FOR SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 155 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro Glu Asp Gly
1               5                   10                  15
Gly Ser Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro Lys Arg Leu
            20                  25                  30
Tyr Cys Lys Asn Gly Gly Phe Phe Leu Arg Ile His Pro Asp Gly Arg
        35                  40                  45
Val Asp Gly Val Arg Glu Lys Ser Asp Pro His Ile Lys Leu Gln Leu
    50                  55                  60
Gln Ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val Cys Ala Asn
65                  70                  75                  80
Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu Ala Ser Lys Cys
                85                  90                  95
Val Thr Asp Glu Cys Phe Phe Phe Glu Arg Leu Glu Ser Asn Asn Tyr
            100                 105                 110
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Asn Thr Tyr Arg Ser Arg Lys Tyr Thr Ser Trp Tyr Val Ala Leu Lys
115 120 125

Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro Gly Gln Lys
130 135 140

Ala Ile Leu Phe Leu Pro Met Ser Ala Lys Ser
145 150 155

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Ala Gly Ser Ile Thr Thr
1 5

(3) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Ala Gly Ser Ile Thr Thr
1 5

(4) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRITPION: SEQ ID NO:4:

Ala Gly Ser Ile Thr Thr
1 5

(5) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Ser Ile Thr Thr
1 5

(6) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Ile Thr Thr
1

Claims

1. A process for the preparation of a basic fibroblast growth factor (bFGF) which process comprises:

   (i) forming an adduct between heparin or heparan sulphate and a bFGF which has the 9-10 Leu-Pro bond;
   (ii) treating the adduct with pepsin A or cathepsin D, thereby cleaving the said bond; and
   (iii) releasing from the adduct the bFGF thus obtained.

2. A process according to claim 1, wherein a mixture of two or more said bFGFs whose amino acid sequences differ only by having different N-termini is employed in step (i).

3. A process according to claim 2, wherein the said mixture is composed of bFGFs having the general formula:

$$NH_2\text{-(AA)}_x\text{-Leu-Pro-Y-COOH}$$

wherein AA is any amino acid residue, x is zero or an integer and Y denotes the amino acid sequence of (11-l55) bFGF.

4. A process according to claim 3, wherein $(AA)_x$ denotes a sequence shown in one of SEQ ID Nos. 2 to 6, IleThrThr, ThrThr or Thr.

5. A process according to claim 3, wherein the said mixture is composed of (2-155)bFGF and (3-155)bFGF.

6. A process according to any one of the preceding claims, wherein the or each bFGF is a human, murine or rodent bFGF.

7. A process according to any one of the preceding claims, wherein the heparin or heparan sulphate and the or each bFGF are provided in a buffered aqueous solution in step (i) and the pepsin A or cathepsin D is added to the resulting solution in step (ii).

8. A process according to any one of claims 1 to 6, wherein the heparin or heparan sulphate is immobilised on a support to form an affinity column, a solution of the said bFGFs is loaded onto the column in step (i), a solution of the pepsin A or cathepsin D is passed through the thus loaded column in step (ii) and the truncated single form of bFGF thus obtained is eluted from the column in step (iii).

Revendications

1. Procédé de préparation d'un facteur de croissance fibroblastique basique (bFGF), lequel procédé comprend les opérations consistant à :

   (i) former un produit d'addition entre l'héparine ou l'héparan sulfate et un bFGF qui a la liaison Leu-Pro 9-10 ;

   (ii) traiter le produit d'addition par de la pepsine A ou de la cathepsine D, clivant ainsi ladite liaison ; et

   (iii) libérer du produit d'addition le bFGF ainsi obtenu.

2. Procédé selon la revendication 1, dans lequel un mélange d'au moins deux desdits bFGF, dont les séquences d'acides aminés diffèrent uniquement en ayant des extrémités N-terminales différentes, est employé à l'étape (i).

3. Procédé selon la revendication 2, dans lequel ledit mélange est composé de bFGF ayant la formule générale :

$$NH_2\text{-(AA)}_x\text{-Leu-Pro-Y-COOH}$$

dans laquelle :

- AA représente n'importe quel reste d'acide aminé ;
- x est zéro ou un nombre entier ; et
- Y désigne la séquence d'acides aminés de (11-155)bFGF.

4. Procédé selon la revendication 3, dans lequel $(AA)_x$ désigne une séquence représentée dans l'un parmi SEQ ID N° 2 à 6, IleThrThr, ThrThr ou Thr.

5. Procédé selon la revendication 3, dans lequel ledit mélange est composé de (2-155)bFGF et (3-155)bFGF.

6. Procédé selon l'une des revendications précédentes, dans lequel le ou chaque bFGF est un bFGF humain, murin ou de rongeur.

7. Procédé selon l'une des revendications précédentes, dans lequel l'héparine ou l'héparan sulfate et le ou chaque bFGF sont mis en oeuvre sous forme d'une solution aqueuse tamponée à l'étape (i) et la pepsine A ou la cathepsine D est ajoutée à la solution résultante à l'étape (ii).

8. Procédé selon l'une des revendications 1 à 6, dans lequel l'héparine ou l'héparan sulfate est immobilisé sur un support pour former une colonne d'affinité, une solution desdits bFGF est chargée sur la colonne à l'étape (i), une solution de la pepsine A ou de la cathepsine D est amenée à passer à travers la colonne ainsi chargée à l'étape (ii), et la forme simple tronquée de bFGF ainsi obtenue est éluée de la colonne à l'étape (iii).

Patentansprüche

1. Verfahren zur Herstellung eines basischen Fibroblasten-Wachstumsfaktors (bFGF), umfassend:

   (i) Ausbilden eines Adduktes zwischen Heparin oder Heparansulfat und einem bFGF mit 9-10 Leu-Pro-Bindung;
   (ii) Behandeln des Adduktes mit Pepsin A oder Kathepsin D, wodurch diese Bindung gespalten wird; und
   (iii) Freisetzen des so erhaltenen bFGF aus dem Addukt.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß in Stufe (i) eine Mischung aus zwei oder mehreren der bFGFs, dessen Aminosäuresequenzen sich nur durch verschiedene N-Termini unterscheiden, verwendet wird.

3. Verfahren nach Anspruch 2, dadurch gekennzeichnet, daß die Mischung aus bFGFs der allgemeinen Formel:

$$NH_2\text{-}(AA)_x\text{-Leu-Pro-Y-COOH}$$

zusammengesetzt ist, worin AA irgendein Aminosäurerest ist, x 0 oder eine ganze Zahl bedeutet und Y die Aminosäuresequenz von (11-155)bFGF darstellt.

4. Verfahren nach Anspruch 3, dadurch gekennzeichnet, daß $(AA)_x$ eine in einer der SEQ ID No. 2 bis 6 dargestellte Sequenz, IleThrThr, ThrThr oder Thr darstellt.

5. Verfahren nach Anspruch 3, dadurch gekennzeichnet, daß sich die Mischung aus (2-155)bFGF und (3-155)bFGF zusammensetzt.

6. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß der oder jeder bFGF ein bFGF von Menschen, Mäusen oder Nagetieren ist.

7. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß das Heparin oder Heparansulfat und der oder jeder bFGF in Stufe (i) in einer gepufferten wässerigen Lösung bereitgestellt werden und das Pepsin A oder Kathepsin D in Stufe (ii) zu der resultierenden Lösung zugegeben wird.

8. Verfahren nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß das Heparin oder Heparansulfat an einem Träger immobilisiert ist, um eine Affinitätssäule auszubilden, in Stufe (i) eine Lösung der bFGFs auf die Säule aufgebracht wird, in Stufe (ii) eine Lösung von Pepsin A oder Kathepsin D durch die so beschickte Säule hindurchgeführt wird, und die so erhaltene abgestumpfte Einzelform von bFGF in Stufe (iii) eluiert wird.